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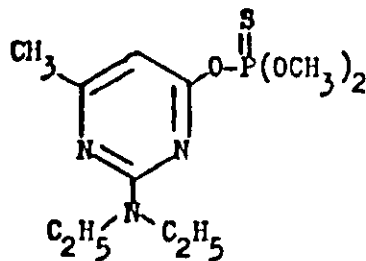
PFRAM - 11A

RESIDUE ANALYTICAL METHOD NO. 11A

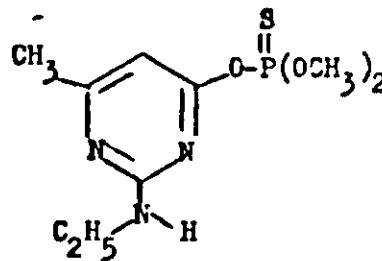
DETERMINATION OF RESIDUES OF PIRIMIPHOS-METHYL
AND ITS PHOSPHORUS-CONTAINING METABOLITES IN CROPS
WATER, MILK AND ANIMAL TISSUES

1. SCOPE

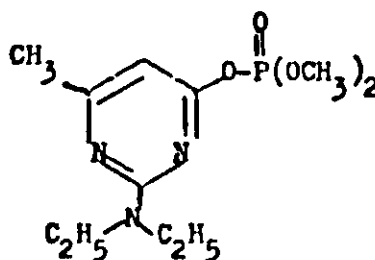
The method is suitable for the determination of the residues of pirimiphos-methyl (i) in crops, water, milk and animal tissues. The method also determines the phosphorus-containing metabolites (ii, iii) which are detected occasionally as very minor metabolites in some crop samples. The limit of detection of the method is generally 0.01 ppm.



(i)



(ii)



(iii)

2. METHOD SUMMARY

Crop samples are extracted with 20% acetone in n-hexane. A decanted portion of the extract is washed with water, and an aliquot injected into a gas chromatograph equipped with a flame photometric or thermal ion

detector. The size of peaks obtained at the retention times of piriphos-methyl and its metabolites are used to calculate the residues present. Animal tissues are extracted by boiling with n-hexane and milk is extracted with a mixture of acetone and acetonitrile. Oily or fatty materials are subjected to a cleanup by acetonitrile-n-hexane partition before analysis.

3. REAGENTS

- (a) Acetone redistilled if necessary.
- (b) n-Hexane " " "
- (c) Methanol " " "
- (d) Acetonitrile " " "
- (e) Florisil 60-100 mesh (British Drug Houses Ltd.).
- (f) Celite 545 filter aid (Phase Separations Ltd.).
- (g) Chromosorb W-HP 80-100 mesh (Phase Separations Ltd.).
- (h) Phenyl-diethanolamine succinate (Phase Separations Ltd.).
- (i) Fluorosilicone Oil QFI (Phase Separations Ltd.).
- (j) Silicone Gum Rubber S-30 Ultra-phase (Phase Separations Ltd.).
- (k) Samples of piriphos-methyl and its metabolites of known purity. (Store in dark under refrigeration).
- (l) Gases for GLC, pure nitrogen (e.g. 'white spot' BOC) hydrogen, air oxygen.

NOTE: Solvents used in the analysis should be examined for possible interfering impurities by concentrating 100 ml down to 5 ml and injecting a 5 µl aliquot into the gas chromatograph.

Other reagents used should also be carefully checked for interfering impurities.

4. APPARATUS

- (a) Laboratory chopper used for initial preparation of samples (e.g. Hobart model).
- (b) High-speed macerator or blender (e.g. Sorvall Omni-Mixer Homogenizer available from V.A. Howe and Co. Ltd., London, U.K. or I. Sorvall Inc., Norwalk, Connecticut, U.S.).
- (c) Rotary evaporator or Kugelrohr-Danish concentrator, fitted with 3 ball Snyder column.
- (d) Water bath capable of maintaining a temperature of 30°C.
- (e) Syringes for GLC e.g. 10 µl Hamilton 701 SN 3".

- (f) Separating funnels 250 ml and 500 ml capacity.
- (g) Glass columns for 'Florisil' cleanup 40 cm x 2.5 cm id. fitted with glass stopcocks.
- (h) Gas-liquid chromatograph equipped with a flame photometric detector or with a suitable thermionic (alkaline flame) detector. The Melpar flame photometric detector is recommended (available from Techmaton Ltd., Middlesex, England).

NOTE: Chromatographs fitted with heated injection ports and on-column injection systems are recommended to avoid decomposition of pirimiphos-methyl. Glass columns and easily interchangeable pre-columns are also desirable.

- (i) Wide mouth 2 oz glass jars fitted with screw capped lids.

5. PROCEDURE

5.1 Gas-Liquid chromatographic conditions.

The following conditions have been found satisfactory when using a Pye 104 gas chromatograph equipped with glass-columns and a heated injection port. The manufacturers manual for the gas chromatograph concerned should be consulted for details concerning amplifier and recorder settings.

- (a) Column.
 - 90 cm long x 3 mm internal diameter glass.
- (b) Column packing 3% w/w phenyldiethanolamine succinate on Chromosorb W-HP (80-100 mesh).
- (c) Alternative column packing
 - (i) 1:1 mixture of 4% SE-30 (ultraphase) - 6% QFI on Chromosorb W-HP (80-100 mesh).
 - (ii) 7% QFI on Chromosorb W-HP (80-100 mesh).
- (d) Confirmatory column packing.
 - 270 cm long x 3 mm internal diameter.
 - 3% SE-30 (ultraphase) on Chromosorb W-HP 80-100 mesh.
- (e) Column Temperatures

(i) 3% PDEAS	180°C	{ for 90 cm column }
(ii) 4% SE-30/6% QFI	210°C	
(iii) 7% QFI	160°C	
(iv) 3% SE-30	220°C	{ for 270 cm column }
- (f) Injection Port Temperature.
 - 15°C above column temperature.
- (g) Carrier gas flow rate
 - Pure dry nitrogen at 100 ml/minute.

(h) Detector.

Melpar flame photometric detection with phosphorus filter (526 nm filter). The setting up of the flame photometric detector should be carried out as detailed in the manufacturers literature. This detector is usually operated at 15-20°C above the column temperature, and with gas flows as follows: hydrogen 200 ml/minute, oxygen 35 ml/minute, air 25 ml/minute.

The hydrogen/oxygen gas flow ratio should be adjusted until a satisfactory response is obtained for pirimiphos-methyl and its metabolites.

(i) Retention Times.

The retention times given below should be regarded as approximate values and will vary slightly according to the age and condition of the column.

(i) 3% PDEAS	pirimiphos-methyl	1.9 minutes.
	(ii)	3.9 minutes.
	(iii)	2.7 minutes.
(ii) 7% QFI	pirimiphos-methyl	2.0 minutes.
	(iii)	3.0 minutes.
	(ii)	2.4 minutes.
(iii) 3% SE-30	pirimiphos-methyl	6.5 minutes.
(270 cm column)	(ii)	5.9 minutes.
	(iii)	6.0 minutes.

(j) Column Preparation.

(i) The following method is recommended, using the preparation of the 3% PDEAS column packing as an example.

Dissolve 0.3 g phenyldiethanolamine succinate in acetone (100 ml) and add 10 g Chromosorb W-HP (80-100 mesh) to this solution contained in a round bottomed flask (250 ml). Remove the majority of the solvent under vacuum on a rotary evaporator until a soft but not dry mass is produced. Pour the packing on to a petri-dish and allow to air dry overnight.

Thoroughly mix the packing and then pack the column under vacuum with constant vibration, to within 1 cm of the injection port end. Close the column with a silanised glass plug.

Condition the column by installing it in the gas chromatograph unconnected to the detector and heating it overnight at 15°C above its operating temperature.

(ii) The phenyldiethanolamine succinate column gives excellent resolution of pirimiphos-methyl and its metabolites from many other organo-phosphorus insecticides in common use. It does however bleed heavily and has a more limited lifetime than the other recommended column packings.

5.2 Extraction.

(i) Root Crops, Fruit and Vegetables.

- (a) Remove loose soil and roots etc.
- (b) Dice or chop the whole sample thoroughly using a laboratory chopper or mill.

NOTE: If samples have been deep frozen while awaiting analysis, they should be sub-sampled while only partially thawed, to avoid loss of liquid. In some cases it may be desirable to add solid carbon dioxide to the sample during mincing or chopping.

(c) Mix the chopped sample and weigh out a representative portion (100 g) into a macerating jar. Add 400 ml of 20% acetone in n-hexane and macerate at high speed for 10 minutes. Allow the plant debris to settle by standing for 30 minutes and then decant a portion of the supernatant liquid (approximately 50 ml) into a separating funnel (250 ml).

(d) Add an equal volume (~50 ml) of water to the decanted extract in the separating funnel, shake together for 1 minute and when the two layers have separated drain off the lower water layer including any interfacial emulsion. Persistent emulsions should be broken by centrifuging a portion of the emulsified layer and/or by agitation with a glass rod.

(e) Transfer the n-hexane extract to a screw capped air-tight glass bottle. Keep under refrigeration in the dark until required for analysis which should be preferably carried out as soon as possible.

NOTE 1: Mushrooms and similar crops should be extracted with methanol to avoid the persistent emulsions formed on extraction with n-hexane/acetone. A 100 g sub sample is macerated with 400 ml methanol and the macerate filtered through a Whatman No 1 filter paper (9 cm) on a Buchner funnel. The crop debris is sucked dry and the residuum washed with further methanol until the final volume of the filtrate is 500 ml.

NOTE 2: Extracts of crops with n-hexane/acetone, methanol or acetonitrile containing pirimiphos-methyl and its metabolites may be concentrated on a rotary evaporator without loss, provided the temperature of the water bath does not exceed 30°C and the evaporation is slow and gentle. A Kuderna-Danish concentrator is recommended where losses are found to occur. The concentration should not proceed beyond the aqueous residue, owing to the possibility of hydrolysis of the pesticide.

(ii) Stored Grain (Wheat, Barley, Rice, Peanuts), Bread, Bran and Fine Offal.

(a) Thoroughly mix the whole sample and by quartering take a 100 g representative sub-sample. Loaves of bread should be cut up with a knife into 1 inch cubes. When analysing grain it is as well to remember that the majority of the residue is on the outside of the grain and is readily lost as fine dust that may be found adhering to plastic bags or containers.

(b) Grind the sample to a fine powder in a large mill. The temperature of the sample should not be allowed to rise more than 10°C during grinding and precautions should be taken to avoid loss of fine dust.

(c) Weigh a representative aliquot (100 g for grain, 50 g for bread and bran) into a macerating jar and add 50 ml H_2O followed by 400 ml of 20% acetone in n-hexane. Blend the mixture on a high speed macerator for 10 minutes and allow the mixture to stand until a clear aliquot of supernatant liquid (~50 ml) may be poured off into a separating funnel (250 ml). (An aliquot of the mixture may be centrifuged at this point if desired).

(d) Add an equal volume of water to the aliquot portion of extract contained in the separating funnel and shake for one minute.

(e) Allow the n-hexane and water layers to separate and run off the water layer including any interfacial emulsion.

(f) Transfer the n-hexane extract to a screw capped air-tight glass bottle. Keep under refrigeration in the dark until required for analysis which should be preferably carried out as soon as possible.

NOTE 1: Grain and Bread samples can also be extracted with methanol as described for mushrooms under 5.2 (i) & NOTE 1.

NOTE 2: Extracts of grain and bread products can be cleaned-up by following the n-hexane/acetonitrile countercurrent partition method described below for oily or fatty materials.

(iii) Oily or fatty crop samples.

Samples such as olive oil, palm oil, rapeseed oil etc. must be cleaned-up by n-hexane/acetonitrile countercurrent partition before an aliquot of the extract is injected into the gas-liquid chromatograph. Unless this is done the lifetime of the column packing is very short and the response of the detector may be reduced. In certain cases however where the sample contains residues above 1 ppm, sufficient dilution may be possible to avoid clean-up at the discretion of the analyst.

(a) For oily or fatty crops (e.g. rapeseed) carry out the procedure as detailed in 5.2 (i) using 90 ml of the washed n-hexane extract (equivalent to a 25 g crop sample). For samples of oil, dissolve 10 g in 100 ml of n-hexane, warming the mixture if necessary. In both cases then proceed as below.

(b) Extract the n-hexane solution by shaking for 1 minute with 2 x 100 ml portions of acetonitrile in a separating funnel (500 ml), allowing complete separation of the layers each time and rejecting any interfacial emulsion. (The acetonitrile layer is the lower layer).

(c) Combine the acetonitrile layers in a separating funnel (500 ml) and wash with n-hexane (50 ml) by shaking gently for one minute.

(d) Separate the layers and wash with n-hexane by shaking with acetonitrile (30 ml) in a separating funnel (250 ml).

- (e) Reject the n-hexane layer from (d) and add the acetonitrile wash from (d) to the combined acetonitrile layers from (c).
- (f) Concentrate the combined acetonitrile layers to 60 ml (\pm 5 ml) on a rotary evaporator taking great care at this stage to evaporate the excess solvent at a slow and gentle rate and at a temperature not exceeding 30°C.
- (g) Quantitatively transfer the concentrated acetonitrile extract to a separating funnel (500 ml), washing the concentration flask with 10 ml acetonitrile and adding this to the acetonitrile in the separating funnel.
- (h) Add 300 ml of 5% aqueous sodium sulphate to the acetonitrile extract and shake gently. Extract this solution for one minute with 2 x 100 ml portions of n-hexane.
- (i) Allow the layers to separate completely by standing, and combine the upper n-hexane layers, rejecting any small amounts of interfacial emulsion.

Depending on the probable residue levels, this combined n-hexane may be concentrated gently on a rotary evaporator (temperature below 30°C) to a volume suitable for analysis (usually not below 50 ml).

- (j) The final n-hexane solution should be stored under cool dark conditions until required for analysis as detailed in 5.2 (i) e.

(iv) Milk.

- (a) Thoroughly homogenise the milk sample either by vigorous shaking or blending.
- (b) Take a 50 g sub-sample of the homogenised milk and shake vigorously with 50 ml acetone in a macerating jar for 5 minutes.
- (c) Add 200 ml acetonitrile to the acetone-milk mixture and blend on a Sorvall-Omni mixer for 5 minutes.
- (d) Filter the precipitated proteins off through a pad of glass wool supported on a large glass funnel and transfer the filtrate to a separating funnel (500 ml).
- (e) Add 50 ml n-hexane and shake gently for 1 minute. When the two phases have separated, run the bottom acetonitrile layer into a round bottomed flask (500 ml). Reject the top n-hexane wash layer.
- (f) Concentrate the acetonitrile solution down to 60 \pm 5 ml on a rotary evaporator. This process must be carried out very carefully to avoid loss of residue by volatilisation and hydrolysis. The temperature of evaporation should not exceed 30°C and the volume of the concentrate should not on any account go below 60 \pm 5 ml.

(g) Transfer the concentrate quantitatively to a separating funnel (250 ml) with the aid of 2 x 10 water washings of the concentrating flask. Extract the aqueous solution by shaking successively with 100 ml and 50 ml portions of n-hexane.

(h) Concentrate the combined n-hexane extracts and washings on a rotary evaporator (temperature $< 30^{\circ}\text{C}$) to approximately 7 ml. Transfer the concentrate to a 10 ml volumetric flask and make up to the mark using n-hexane washings of the concentration flask.

(i) Store the solution under refrigeration at 0°C until required for analysis.

(v) Animal Tissue.

(a) Allow the sample to partially thaw without loss of fluid (i.e. blood).

(b) Finely mince or chop the sample, adding solid CO_2 if necessary during this process to avoid thawing.

(c) By quartering take a 50 g sub-sample and grind in a large pestle and mortar with 50 g sharp quartz sand and sufficient anhydrous granular sodium sulphate to give a uniform dry mixture.

(d) Transfer the mixture quantitatively to a large beaker (500 ml), add 200 ml n-hexane and warm gently with stirring on a water bath until the solvent boils gently. Allow to simmer gently for 1 minute and then decant the solution through a Whatman No. 1 filter paper into a measuring cylinder (250 ml). Extract the residue in the beaker again by washing with 50 ml n-hexane, finally transferring the tissue residue to the filter paper and washing with sufficient n-hexane to make the volume of n-hexane extract in the measuring cylinder up to 250 ml (when cool).

(e) Transfer a 50 ml aliquot of the n-hexane extract to a separating funnel (250 ml) together with 100 ml acetonitrile and shake vigorously for 1 minute. Allow the layers to separate and run the bottom acetonitrile layer into another separating funnel (250 ml). Wash the acetonitrile solution by shaking for 1 minute with 50 ml n-hexane. Again allow the layers to separate, and run the lower acetonitrile layer into a round bottomed flask.

NOTE 1: The sample size is reduced to 10 g by taking a 50 ml aliquot for analysis.

NOTE 2: Hexane extracts of samples such as mutton fat, beef suet etc. may be cleaned up before acetonitrile partition by allowing the n-hexane extract to stand overnight under refrigeration at 0°C . This causes the

bulk of the fat to precipitate, and the 50 ml aliquot for analysis is removed by pipette whilst the solution is still cold.

(f) Extract the combined n-hexane washings by shaking for 1 minute with 50 ml acetonitrile in a separating funnel. Combine all the acetonitrile extracts.

(g) Concentrate the combined acetonitrile extracts carefully and slowly down to approximately 7 ml on a rotary evaporator, keeping the temperature below 30°C.

(h) Transfer the acetonitrile concentrate quantitatively to a 10 ml volumetric flask, and make up to the mark with acetonitrile washings of the concentration flask. Store this solution under refrigeration until required for analysis. It should be clear and pale yellow in colour.

NOTE: Any concentration of solutions containing pirimiphos-methyl and its metabolites should be checked for losses by volatilisation and/or hydrolysis.

A Kuderna-Danish concentrator may be necessary for concentration down to low volumes.

5.3 Determination

(a) Prepare a series of standard solutions of pirimiphos-methyl and metabolites in n-hexane to cover the residue range expected. For crops a range of concentrations from 5 µg to 500 µg in 320 ml n-hexane is normally sufficient.

(b) Make a number of 5 µl injections of the strongest standard into the gas chromatograph until a constant response is obtained. This 'priming' of the column may need to be repeated throughout the lifetime of the column.

NOTE: A 10 µl syringe is recommended for injections of solutions into the gas chromatograph. To obtain satisfactory results using peak height measurements, the method of injection used by each operator should be standardised. 5 µl injections are recommended but this may need to be reduced to 2-3 µl for thermionic detectors. After filling the syringe it should be free from air bubbles and the tip should be wiped clean with tissue paper. The plunger should be depressed rapidly and the needle allowed to remain in the injection port for 3 seconds before being withdrawn.

(c) Make a number (up to 6) repetitive injections of the standard solution whose concentration approximates to that of the sample extract and check that good replication of peak height and retention time is obtained. Variations of more than 5% in peak height and more than 0.2 minute in retention time make the injection suspect. Attention should then be given to operating parameters such as gas leaks, syringe faults or contaminated column packings. Gas chromatographs equipped with thermionic detectors (alkaline flame) are more prone to variations than

those equipped with flame photometric detectors. Where a thermionic detector is being used it is recommended that a standard solution is injected after every third sample injection to check the detectors response.

(d) Prepare a calibration graph from the standard solutions showing $\mu\text{g/ml}$ pirimiphos-methyl and metabolites versus detector response expressed as peak height multiplied by attenuation of the gas chromatograph amplifier. Alternatively 'bracket' the response given by an injection of the sample extract by injecting two standards of the appropriate strengths.

(e) Inject $5 \mu\text{l}$ of the extract prepared from a 'control' untreated or 'check' sample. Examine the chromatogram carefully to check that the control extract is free from any extraneous peaks occurring at the retention time of pirimiphos-methyl and metabolites. The attenuation of the amplifier should be set, during this process, at the sensitivity likely to be used in the analysis. Check also that injection of control extracts do not give increased noise level, rising base lines, excessive 'solvent' responses, negative responses or peaks of long retention time which would interfere with subsequent analysis.

Observation of any response of this nature, usually means heavy and unacceptable contamination with sample extracts, lipids, sterols or plasticizers, such as dibutyl phthalate. The samples for analysis must then be subjected to further clean-up before GLC determination.

(f) Inject an aliquot (usually $5 \mu\text{l}$) of the sample extract into the GLC using the same technique as described for the injection of the standards. If a peak occurs at the retention time of pirimiphos-methyl or its metabolites, repeat the injection and carefully compare the retention time of the peaks to that of the standard solutions. If the identification is doubtful change the column packing to one of those described under section 5.1 (d) and repeat the injection. Retention times should not differ by more than 0.2 minute. If the repeatability of peak heights, retention times and identification is satisfactory, use the measurement of peak height of sample compared with standards to calculate the residue present using the simple formula below:

$$R = \text{ppm residue} = \frac{W}{S}$$

where W = amount of pirimiphos-methyl and metabolites in micrograms (μg) found in aliquot of sample injected (from standards)

S = sample weight (grams) represented by aliquot injected.

(g) The recovery factor to be applied in calculation of the actual residue present in any particular sample should be obtained by each

operator by fortifying untreated control samples at that residue level likely to be found in the actual field samples. Where residues of pirimiphos-methyl and metabolites are very low or undetectable, recovery experiments should be carried out by fortifying control untreated samples at, or near, the claimed residue detection limit.

(h) Recoveries in crop samples at the 0.1 ppm - 5 ppm level are usually in the range $90 \pm 10\%$. Recoveries in samples requiring cleanup by acetonitrile-n-hexane partition such as milk and animal tissues are usually of the order $80 \pm 10\%$. Higher recoveries in these samples can be obtained if desired at the expense of a longer method, by increasing the number of counter-current extractions in the n-hexane-acetonitrile cleanup stage.

5.4 Limits of Detection.

(a) The limits of detection obtained in the residue method depend obviously on the sensitivity of the detector and concentration of the sample extract. Defining the limit of detection as 4 x noise level of amplifier at the maximum attenuation (sensitivity) used, limits of detection of 0.01 ppm for crops and 0.005 ppm for milk can usually be obtained without difficulty.

5.5 General Notes.

(i) The top 7 cm of the column packing which is the injection zone for on-column injection chromatographs may need replacing regularly. This is especially the case for uncleared-up crop extracts. Pre-conditioned column packing or silanized glass wool may be used for this purpose.

(ii) Injections of extracts of samples containing a high residue should be followed by injections of pure solvents (acetone or n-hexane) to avoid spurious residues due to 'ghosting'.

(iii) Fortifications of crop samples for recovery experiments should be carried out by spiking macerated crop samples with solutions of pirimiphos-methyl and metabolites in acetone.

(iv) Residues of pirimiphos-methyl and metabolites on crops are stable under the conditions of storage normally employed (deep freeze at -14°C).

(v) Analysis of water samples can be accomplished by extraction of the sample with n-hexane. Pirimiphos-methyl and its metabolites are readily hydrolysed under aqueous conditions (Half-life of approx. 1 day in sunlight under neutral conditions).

They are also readily absorbed from aqueous solution by plastic containers. Water samples should therefore be stored in aluminium containers under refrigeration.

(vi) While the residue method has been developed for pirimiphos-methyl and its two potential organo-phosphorus containing metabolites, (ii) and (iii), a very large number of residue analysis on a wide variety of samples has indicated that significant residues of (ii) and (iii) are

rarely found. For many samples therefore the residue method is simplified to being determination of pirimiphos-methyl only.

(vii) Organic solvents containing pirimiphos-methyl and its metabolites should not be dried with large amounts of anhydrous sodium sulphate since losses by adsorption can occur, particularly from n-hexane extracts.

(viii) An additional cleanup stage using solid-liquid chromatography on Florisil (60-100 mesh) may be employed if necessary following n-hexane-acetonitrile partitioning as described below.

(a) Preparation of packing.

Heat the Florisil overnight at 650°C and allow to cool in a vacuum desiccator. Store at 130°C until required for de-activating. De-activate each batch before preparing columns by adding 12% water and mixing on a roller mill for 1 hour before use.

(b) Preparation of column.

Add 20 g de-activated Florisil to a glass chromatographic column (40 cm x 2.5 cm i.d.) followed by 15 g of anhydrous granular sodium sulphate.

(c) Transfer the n-hexane extract following the acetonitrile counter-current cleanup (sections 5.3 (iii) d) to the column and elute the residues by collecting successively and separately 100 ml portions of n-hexane-diethyl ether (7:3) and then diethylether-acetone (9:1).

Pirimiphos-methyl is eluted in the 100 ml portion of n-hexane-ether (7:3) while the metabolites (ii) and (iii) elute in 100 ml ether-acetone (9:1).

(d) The eluents may then be concentrated separately for GLC determination.

NOTE : The activity of the Florisil may vary slightly with the crop sample and should be checked accordingly, by eluting a fortified extract with the appropriate solvents and analysing 10 ml fractions for compound recovery.